

# Detection of NADH and ethanol at a graphite electrode modified with titania sol-gel/Meldola's Blue/MWCNT/Nafion nanocomposite film

## Research Article

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**Abstract:** For electrocatalytic determination of NADH, a graphite electrode modified with titania sol-gel/Meldola's Blue/MWCNT/Nafion nanocomposite was proposed. The composition of the matrix film was optimised in terms of the content of carbon nanotubes and Nafion. Incorporation of a redox mediator, Meldola's Blue, into the nanocomposite film enabled electrocatalytic determination of NADH at a low potential, -50 mV. For determination of ethanol, alcohol dehydrogenase (ADH) was immobilized into the matrix layer. Experimental conditions affecting the biosensor response were examined, including enzyme loading, temperature of measurement and pH of background electrolyte. Assessments of the analytical characteristics of the biosensor were performed with respect to sensitivity, limit of detection, operational stability, repeatability and reproducibility. The proposed biosensor showed electrocatalytic activity toward oxidation of ethanol with sensitivity of  $2.24 \mu\text{A L mmol}^{-1}$ , linear range from 0.05 to 1.1 mmol L<sup>-1</sup>, and limit of detection of 25  $\mu\text{mol L}^{-1}$ . The apparent Michaelis-Menten constant was 1.24 mmol L<sup>-1</sup>, indicating a high biological affinity of ADH/titania sol-gel/Meldola's Blue/MWCNT/Nafion electrode for ethanol. The developed biosensor was tested in determinations of ethanol content in alcoholic beverages.

**Keywords:** MWCNT • Titania sol-gel • Nafion • NADH • ADH

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## 1. Introduction

The determination of alcohol content for control of the fermentation process and resultant product quality is crucial to the food and beverage industries [1]. Besides these commercial applications, quantitative measurement of alcohol concentration is also significant in clinical, agricultural and environmental analyses [2]. Among methods reported to determine the content of ethanol, the most widely used are chromatography, spectrometry and refractometry [1,3]. These methods, however, are expensive, time-consuming and sometimes include complicated sample pre-treatment. Electrochemical methods for alcohol determination, especially those employing amperometric biosensors, have been regarded as promising because of their effectiveness, simplicity and selectivity. Enzymatic methods of alcohol determination are based on alcohol oxidase (AOX) or alcohol dehydrogenase (ADH), which catalyse the conversion of alcohol to aldehyde, the latter requiring

nicotine adenine dinucleotide (NAD<sup>+</sup>) as a cofactor. An analytical signal emerged as a result of oxidation of a reduced form of the cofactor NADH is generated during the enzymatic reaction of analyte consumption [4]. A large overpotential encountered in NADH oxidation at conventional bare electrode surfaces potentially leads to the oxidation of other electroactive species present in the media. Many efforts have been made to overcome this problem. Amongst others, the application of redox mediators with low formal potentials has been proposed. Phenantroline [5,6] and phenothiazine derivatives [3,7], phenoxazine dyes [8-10] and redox polymers [11,12] have been used in the construction of ethanol biosensors for the electrocatalytic oxidation of NADH at low potential. Recently, modification of the biosensor matrix with nanomaterials was reported. An alteration of this type usually improves transfer of electrons between the sensor matrix and the bioelement. Carbon nanotubes (CNTs) constitute attractive components of the immobilization layer due to their high chemical and mechanical stability,

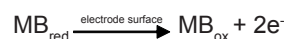
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excellent electronic properties and high surface area. There have been several reports on ethanol biosensors based on composites of carbon nanostructured materials and redox mediators. Jiang *et al.* [9] proposed an ethanol biosensor based on integration of ADH with Meldola's Blue/ordered mesoporous carbon electrode. A composite of CNT, glutaraldehyde, graphite powder, redox mediator Meldola's Blue and ADH was used by Santos *et al.* for preparation of alcohol biosensor [10]. Du *et al.* [12] employed single-walled carbon nanotubes (SWCNT) functionalised with poly(nile blue A) in the construction of a dehydrogenase-based biosensor. The application of biosensors based on ADH entrapped in composite consisted of colloidal nanogold, multi-walled carbon nanotubes (MWCNT) and Teflon for determination of ethanol in alcoholic beverages was reported by Manso *et al.* [13]. Electrocatalytic biosensing of ethanol was performed using a glassy carbon electrode modified with carbon nanotubes prepared by noncovalent functionalisation with 1,10-phenantroline-5,6-dione [5]. Analytical characteristics of some mediator and CNT-based biosensors proposed for determination of ethanol content are shown in Table 1.

Our previous study dealt with NADH sensors based on a redox mediator, Meldola's Blue, entrapped on the surface of graphite electrode using a titania layer obtained by sol-gel process [4]. The results of experiments suggested that Meldola's Blue incorporated into the composite successfully lowered the potential of NADH electrooxidation, allowing NADH detection at the low value of -50 mV vs. Ag/AgCl reference electrode.

The aim of this work was to develop an amperometric biosensor for ethanol determination based on ADH entrapped in a composite of titania sol-gel modified

with multi-walled carbon nanotubes (MWCNT), Meldola's Blue and Nafion. Titania sol-gel was chosen as a binding agent of biosensor matrix. Sol-gel films, compared with other immobilization matrices, have many advantages such as entrapment of large amounts of enzymes, thermal and chemical stability and simple preparation without covalent modification [14]. Nafion, perfluorinated sulfonate ionomer, can absorb MB through ion exchange [15], moreover it was reported as a component that decreased cofactors leakage and simultaneously enhanced the sensor stability [16]. Meldola's Blue, strongly assembled on the surface of CNT by non-covalent  $\pi$ - $\pi$  stacking [17], decreases the deactivation of ADH [18] and acts as a redox mediator in the oxidation of NADH, which is produced during the enzymatic conversion of alcohol:



The NADH sensor matrix composition was optimised with respect to MWCNTs and Nafion, and the analytical characteristics of the resulting sensor was performed. Incorporation of these two components into the titania sol-gel/MB matrix significantly improved responses of the modified electrode to NADH when compared to the results of our earlier work [4], and to other carbon composite sensors reported in literature. For development of ethanol-biosensor, loading of ADH per electrode, as well as monitoring and controlling experimental conditions such as temperature of measurement and

**Table 1.** Comparison of analytical characteristics of mediator and CNT-based ADH biosensors for ethanol.

| Immobilization matrix             | Working potential (mV) | Linear range (mmol L <sup>-1</sup> ) | Sensitivity ( $\mu\text{A L mmol}^{-1}$ )            | LOD ( $\mu\text{mol L}^{-1}$ ) | Ref.      |
|-----------------------------------|------------------------|--------------------------------------|--|--------------------------------|-----------|
| OMC/MB                            | -100                   | up to 6                              | 0.0346   | 19                             | [9]       |
| MWCNT/MB                          | 0                      | 0.05–10                              | 4.75 $\mu\text{A L mmol}^{-1} \text{cm}^{-2}$        | 6                              | [10]      |
| PD/MWCNT                          | 0                      | up to 7                              | 0.01085  | 300                            | [5]       |
| CA-TBO                            | -400–0*                | 0.01–0.4                             | 0.41   | 5                              | [3]       |
| TTF-Cys SAM                       | 100                    | 1–10                                 | 0.0435   | 30                             | [1]       |
| SWCNT/PBCB                        | 0                      | 0.4–4.2                              | -----  | 100                            | [8]       |
| Au <sub>coll</sub> /MWCNT/Teflon  | 300                    | 0.02–1                               | 2.27   | 4.7                            | [13]      |
| PNb/SWCNT                         | 100                    | 0.1–3.0                              | -----  | 50                             | [12]      |
| TiO <sub>2</sub> /MWCNT/Nafion/MB | -50                    | 0.05–1.1                             | 2.24<br>8.0 $\mu\text{A L mmol}^{-1} \text{cm}^{-2}$ | 25                             | This work |

\* differential pulse voltammetry; Au<sub>coll</sub> – colloidal nanogold; CA – cellulose acetate; Cys – cysteamine; MB – Meldola's Blue; MWCNT – multi-walled carbon nanotubes; OMC – ordered mesoporous carbon; PBCB – poly(brilliant cresyl blue); PD – 1,10-phenantroline-5,6-dione; PNb – poly(nile blue A); SAM – self-assembled monolayer; SWCNT – single-walled carbon nanotube; TBO – toluidine blue O; TTF – tetrathiafulvalene.

pH of background electrolyte, were performed. The biosensor formulated using this technique exhibited satisfactory analytical characteristics and sensitivity. For verification purposes, the functioning of the biosensor was successfully tested in determination of ethanol content in alcoholic beverages.

## 2. Experimental procedure

### 2.1. Chemicals

All chemicals were analytical-grade reagents. Solutions were prepared using double-distilled water. Titanium(IV) isopropoxide, multi-walled carbon nanotubes MWCNT (O.D. 10-15 nm, I.D. 2-6 nm, length 0.1-10  $\mu\text{m}$ , >90% purity), Meldola's Blue (MB),  $\beta$ -NADH,  $\beta$ -NAD<sup>+</sup> and *Saccharomyces cerevisiae* alcohol dehydrogenase ADH (75 KU) were purchased from Sigma (USA); ethanol (96%), 2-propanol, HCl (35%) and L-(+)-ascorbic acid were from POCh (Poland); HNO<sub>3</sub> (65%), NH<sub>3(aq)</sub> (25%) and acetone were obtained from LACHNER (Czech Republic); 0.3  $\mu\text{m}$  alumina, used for polishing working electrode surfaces, was from Buehler Micropolish (USA); Nafion, perfluorinated sulfonate ionomer, 5% (w/v) solution in mixture of low aliphatic alcohol and water, was purchased from Fluka. Buffer solutions (all in concentration of 0.1 mol L<sup>-1</sup>) were prepared by mixing appropriate volumes of KH<sub>2</sub>PO<sub>4</sub> and Na<sub>2</sub>HPO<sub>4</sub> solutions (for pH 7.0 and 8.0, phosphate buffer solutions), or H<sub>3</sub>BO<sub>3</sub> with KCl and NaOH solutions (for pH 9.0, Clark and Lubs buffer solution).

### 2.2. Apparatus and measurements

Electrochemical analyser M161 (mtm-anko, Poland) was employed for the electrochemical measurements. All experiments were performed with a conventional three-electrode system: the graphite working electrode, WE (spectral carbon rod, d=6 mm) covered with nanocomposite layer, the saturated silver/silver chloride reference electrode (RE) (mtm-anko, Poland) and the platinum auxiliary electrode (AE). Amperometric experiments were carried out in an electrochemical cell containing 5 mL of buffer solution under constant stirring with a magnetic bar and under free access of air. The holding potential of working electrode was -50 mV vs. Ag/AgCl (selected during our earlier research, [4]). Determination of ethanol was performed in a 4 mmol L<sup>-1</sup>  $\beta$ -NAD<sup>+</sup> solution.

A Sonic 3 Ultrasonic bath (POLSONIC, Poland) was employed in the sonification procedure, while a combined glass electrode ERH-11 (HYDROMED, Poland) together with a CP-501 pH-meter (Elmetron,

Poland) were employed in pH measurements. A vortex (IKA, Germany) was used to obtain homogenous nanocomposite mixtures.

### 2.3. Preparation of TiO<sub>2</sub>/MB/MWCNT, TiO<sub>2</sub>/MB/MWCNT/Nafion and ADH/TiO<sub>2</sub>/MB/MWCNT/Nafion nanocomposites

Titania sol was prepared by acid hydrolysis and further polycondensation of titanium(IV) isopropoxide as described in our earlier reports [19,20]. Separately, a solution of Meldola's Blue was prepared in 0.1 mol L<sup>-1</sup> phosphate buffer solution at pH 7.0. The concentration of redox mediator in immobilizing composite, 0.125 mmol L<sup>-1</sup>, was chosen based on previous experiments [4]. The titania sol and MB solution were then mixed in a 1:1 ratio.

To the appropriate amount of carbon nanotubes (depending on the number of fabricated sensors) the mixture of sol with MB was gradually added, at which point the composite of sol/MB/MWCNT was shaken. In order to obtain a nanocomposite with Nafion, portions of ionomer were incorporated in the mixture of sol/MB/MWCNT. The resultant composite was sonicated for 10 minutes to obtain a homogenous mixture.

To prepare the enzyme-containing matrix, a solution of alcohol dehydrogenase (9.8 – 49.0 mg mL<sup>-1</sup>) was prepared in 0.5 mol L<sup>-1</sup> phosphate buffer solution, pH 7.0. The ADH solution was mixed with sol/MB mixture, with the composite gradually added to MWCNT and shaken. Next, small portions of Nafion were incorporated and shaken again. Finally, the composite of ADH/TiO<sub>2</sub>/MB/MWCNT/Nafion was sonicated to obtain a homogenate.

### 2.4. NADH sensor and ethanol biosensor fabrication

Optimal (bio)sensor matrix composition and measurement conditions were based on sensitivities obtained, for the sensor, in the NADH solution and, in case of the biosensor, in the ethanol solution.

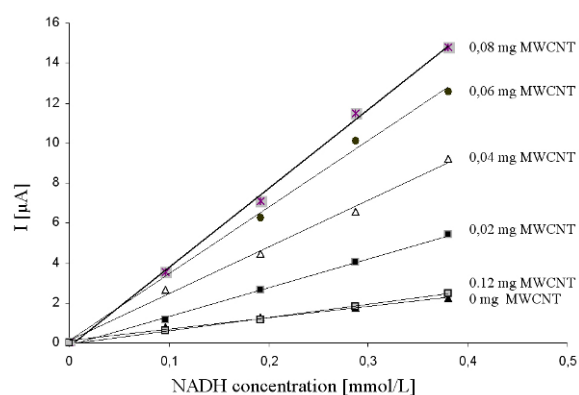
The NADH sensor was firstly optimised with respect to the MWCNT amount per electrode and then Nafion content. In order to construct an ethanol biosensor, the optimum amount of ADH per electrode and pH of the supporting electrolyte were selected.

To prepare each sensor or biosensor, 20  $\mu\text{L}$  (in portion of 10  $\mu\text{L}$ ) of appropriate nanocomposite was deposited on the surface of a pre-treated graphite electrode [19,20]. After each portion of composite had been added, the surface of electrode was dried in air for ca. 10 min. Finally the electrode was allowed to dry over saturated disodium phosphate solution for 20 h at

**Table 2.** Results of ethanol determination in alcoholic beverage samples with ADH/TiO<sub>2</sub>/MB/MWCNT/Nafion biosensor.

| Beverage   | Ethanol (% v/v) |                      |
|------------|-----------------|----------------------|
|            | nominal         | found by biosensor * |
| white wine | 13              | 12.09                |
| red wine   | 11.5            | 11.03                |
| liquor     | 38              | 37.15                |
| vodka      | 40              | 42.80                |

\* mean value of two measurements

**Figure 1.** Dependence of anodic current of NADH sensor on the amount of MWCNT incorporated onto matrix layer;  $E_{app} = -50$  mV vs. Ag/AgCl; supporting electrolyte: 0.1 mol/L phosphate buffer solution, pH 7.0.

4°C. When not in use, sensors were stored at 4°C in phosphate buffer solution, (pH 7.0, 0.1 mol L<sup>-1</sup>), their active surface touching the surface of solution.

## 2.5. Determination of ethanol in alcoholic beverages

Samples of alcoholic beverages were diluted with 0.1 mol L<sup>-1</sup> phosphate buffer solution (pH 8.0) depending on their ethanol concentration: wine samples were diluted threefold while liqueur and vodka tenfold. Analyses were carried out by chronoamperometry in stirred solution at potential of -50 mV vs. Ag/AgCl. In order to eliminate possible matrix effects, determinations were performed using the standard additions method.

## 3. Results and discussion

### 3.1. Sensor for NADH determination

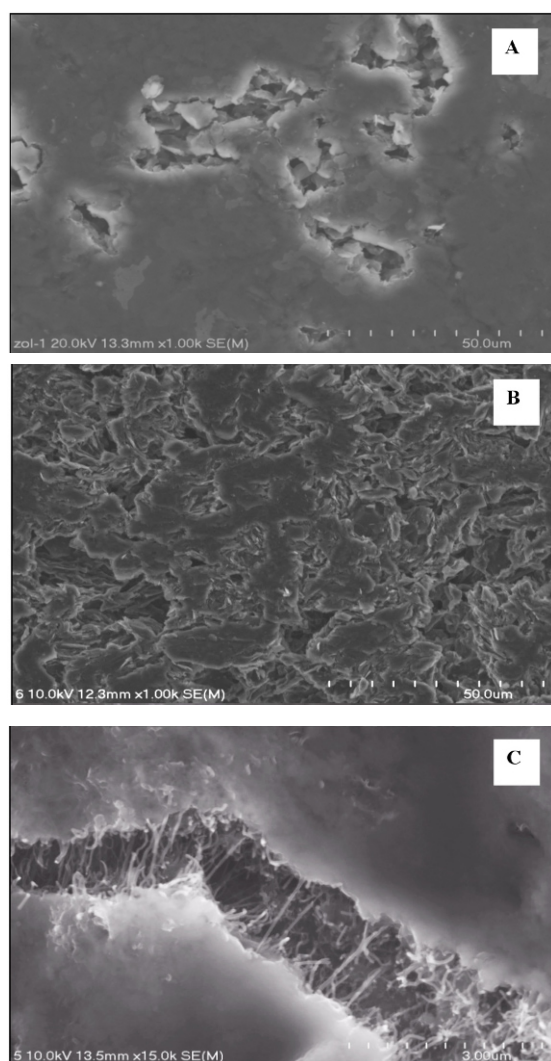
The first step of NADH sensor formulation was optimisation of the amount of carbon nanotubes immobilized on each electrode, with MWCNT loadings tested at 0.02, 0.04, 0.06, 0.08 and 0.12 mg per electrode. For each of these sensors, amperometric

measurements in NADH solutions were performed. As expected, presence of carbon nanotubes considerably affected sensor behaviour, due to their unique properties in promoting electron transfer. From Fig. 1, it may be observed that the highest value of sensor sensitivity was obtained for 0.08 mg of MWCNT entrapped per electrode; immobilization of that amount of carbon nanotubes made the sensitivity six-fold higher than for sensors without carbon nanotubes, 37.5 and 6.2  $\mu\text{A L mmol}^{-1}$ , respectively. The highest studied contents of MWCNT in the matrix layer, 0.12 mg per electrode, led to its cracking and the dropping out of nanocomposite, with the sensor exhibiting low response for NADH. Consequently, 0.08 mg MWCNT per electrode was chosen for further experiments.

The amperometric response was also examined as a function of Nafion content in sensor matrix. Three composites were prepared with following ratios of sol/MB/MWCNT to Nafion mixture: 1:1, 5:2 and 10:1 (v/v). It was observed that the content of ionomer significantly changed the sensor response. The highest value of sensitivity towards NADH occurred for the composite with smallest amount of ionomer compared to sol mixture (sol to Nafion, 10:1, v/v), 21.7  $\mu\text{A L mmol}^{-1}$ . However, for that matrix composition the sensors developed were mechanically less stable. The addition of ionomer (Nafion) into immobilization matrix probably influenced (slowing) NADH diffusion process to electrode surface, which resulting in observed decrease of sensor sensitivity [21]. Nafion forms hydrophilic cation-exchange clusters surrounded partially by hydrophobic fluorocarbon backbone [22,23] and in such a way can increase hydrophobicity of composite. It is possible that the composite hydrophilicity was responsible for worse mechanical stability of sensing layer observed for composite with smaller Nafion content. As a result, the ratio of 5:2 (v/v) titania sol/MB/MWCNT to Nafion was selected. The electrode modified with this matrix composition showed sensor sensitivity of 16.5  $\mu\text{A L mmol}^{-1}$ .

For the selected matrix composition, the sensor displayed a response time of less than 30 seconds and a linear range up to 38 mmol L<sup>-1</sup>. The limit of NADH detection, evaluated according to the equation:  $\text{LOD} = 3 S_x / a$  ( $a$  - mean sensitivity;  $S_x$  - standard deviation of signal in buffer solution,  $n=10$ ) was 7.2  $\mu\text{mol L}^{-1}$ . Comparing the obtained values of sensitivity and LOD to the results of our earlier studies on NADH sensor based on titania sol and MB alone [4], it could be noticed that addition of carbon nanotubes and Nafion to the sensor matrix improved these analytical parameters: for a sensor based on titania sol/MB sensitivity towards NADH and LOD were 12.5 mA L mol<sup>-1</sup> (59 mA L mol<sup>-1</sup> cm<sup>-2</sup>)





**Figure 2.** SEM images of pure  $\text{TiO}_2$  gel (A) and  $\text{TiO}_2$ /MWCNT/Nafion (B,C) nanocomposite (optimal composition) on the surface of graphite electrode.

and  $12 \mu\text{mol L}^{-1}$ , respectively. That phenomenon could be explained by morphology of matrix films. Comparing SEM images of pure titania gel (Fig. 2A) and titania composite with Nafion and carbon nanotubes (Fig. 2B,C) it was clear to see that addition of these compounds changed porosity of matrix layer. In pure titania layer only few pores appeared, while in  $\text{TiO}_2$ /MWCNT/Nafion nanocomposite well distributed three-dimensional micro-porous structure was observed which could facilitate diffusion in matrix layer. Carbon nanotubes due to their unique electric properties promoted electron transfer between substrate and electrode surface improved analytical performance of the sensor.

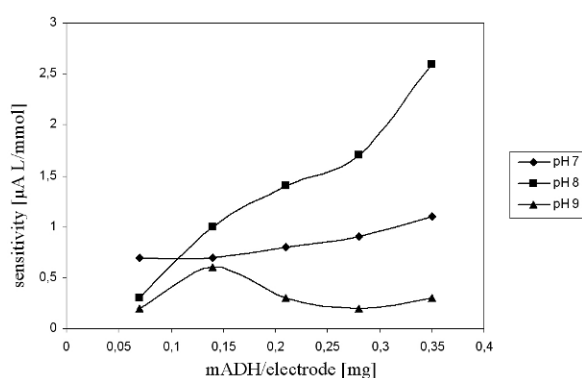
In comparison with other NADH sensors reported recently, the sensitivity of the proposed sensor is higher than that of a sensor based on modified graphene sheet

electrodes [24] with CNT dispersed in a hyaluronic acid [25], based on functionalised CNT incorporating Nile Blue redox mediator [26] and magnetic chitosan microspheres/poly(thionine) modified glassy carbon electrode [27]. Likewise, the limit of NADH detection of developed sensor is lower than reported in [24] and [25]. The results obtained indicate that Meldola's Blue immobilized in a matrix layer acts efficiently as a redox mediator.

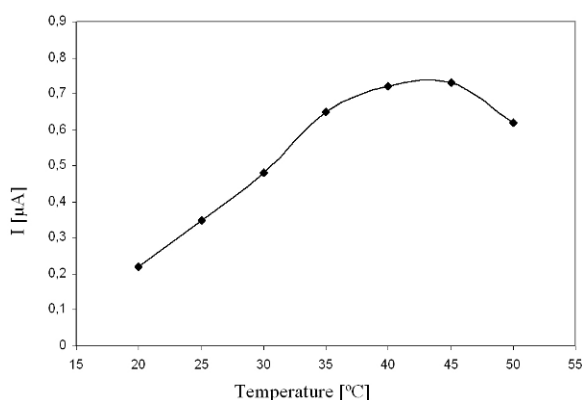
However, there are reports on NADH sensors that exhibited better analytical characteristics. Graphite electrode modified with polyadenylic acid enabled NADH detection at level of  $0.01 \mu\text{mol L}^{-1}$  in concentration range of  $0.025 - 0.1 \mu\text{mol L}^{-1}$  with similar to proposed electrode sensitivity of  $19 \mu\text{A L mmol}^{-1}$  [28]. Manso *et al.* developed sensor based on colloidal gold-carbon nanotubes composite for determination of NADH in linear range  $10 - 1000 \mu\text{mol L}^{-1}$  with sensitivity of  $37.7 \mu\text{A L mol}^{-1}$  [13]. Incorporation of Meldola's Blue into zinc oxide hybrid film resulted in electrode with linear concentration range of  $50 - 300 \mu\text{mol L}^{-1}$ , but higher, than presented sensor, limit of detection:  $10 \mu\text{mol L}^{-1}$  [29]. Similar linear range,  $10 - 300 \mu\text{mol L}^{-1}$ , exhibited glassy carbon electrode covered by poly(*p*-aminobenzene sulfonic acid) (PABS) films doped with flavins [30]. LOD of this sensor was  $1 \mu\text{mol L}^{-1}$ . The ionic liquids/MWCNT/chitosan composite electrode enabled detection of NADH at level of  $0.06 \mu\text{mol L}^{-1}$  with sensitivity of  $84.4 \mu\text{A L mol}^{-1}$  [31].

### 3.2. Optimisation of matrix composition and experimental conditions of ethanol determination

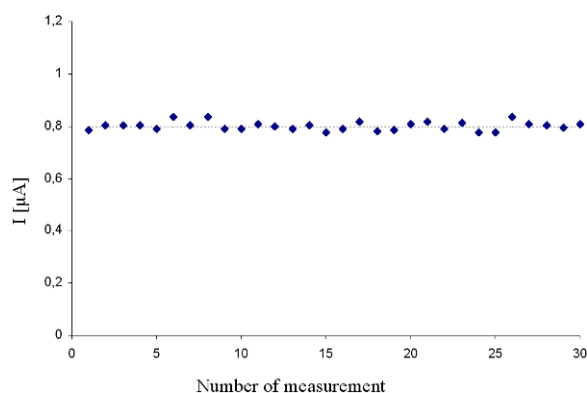
Biosensor performance is strongly connected to enzyme loading. To evaluate this effect, different amounts of enzyme were used in the preparation of the biosensor. For this purpose, bilayers containing 0.07, 0.14, 0.21, 0.28 and 0.35 mg ADH/electrode were prepared, with corresponding ADH activity 78, 156, 234, 312 and  $390 \text{ U cm}^{-2}$  (calculated on the base of initial enzyme activity). As biosensor response is also pH-dependent (because both ADH activity and the MB redox processes are correlated with pH of medium), the choice of medium was performed simultaneously with enzyme loading experiment using buffer solutions of pH 7.0, 8.0 and 9.0. Obtained results are presented in Fig. 3. The strongest effect was observed at pH 8: with quintuple increase of ADH content the sensitivity of biosensor rose nearly eightfold. For a supporting solution of pH 7, the positive influence of amount of ADH was considerable weaker, while in a solution of pH 9, amount of enzyme did not noticeably influence electrode response. Different trends of plots of biosensor sensitivity vs. enzyme



**Figure 3.** Effect of the amount of ADH immobilized into biolayer and pH of supporting electrolyte on sensitivity of ethanol biosensor;  $E_{app} = -50$  mV vs. Ag/AgCl.



**Figure 4.** Effect of temperature on the amperometric response of ethanol biosensor in 0.3 mmol/L solution of ethanol in 0.1 M phosphate buffer solution (pH 8.0) containing 4 mmol/L  $\beta$ -NAD<sup>+</sup>;  $E_{app} = -50$  mV vs. Ag/AgCl.



**Figure 5.** Operational stability of ADH/TiO<sub>2</sub>/MB/MWCNT/Nafion biosensor measured in 0.3 mmol/L ethanol solution, dotted line – mean value of first three measurements; supporting electrolyte: phosphate buffer solution, pH 8.0;  $E_{app} = -50$  mV vs. Ag/AgCl.

loading observed for different pH of background buffers could be caused by disturbing of electron transfer processes in biolayer connected with presence of bulk enzyme molecule that do not possess ability to

conduct electrons [32]. For higher enzyme activity (pH 7 and 8) this process seems to be less significant than simultaneous increment of NADH production connected with increase of enzyme amount. For pH 9, when enzyme activity is significantly reduced (which can be additionally connected with denaturation of biocatalyst molecule) enzyme insulating properties were probably responsible for sensitivity decrease due to increase of the diffusion barrier for the NADH molecule. Consequently, 0.35 mg ADH per electrode ( $390 \text{ U cm}^{-2}$ ) and a supporting electrolyte of pH 8.0 were selected in this study.

Because enzyme activity depends on temperature, the influence of this parameter on biosensor response was studied in the 20–50°C range. According to the results presented in Fig. 4, the optimal temperature was 40°C.

Chronoamperometric measurements of ethanol content were performed under optimum conditions in a solution containing cofactor  $\beta$ -NAD<sup>+</sup> at a fixed concentration of  $4 \text{ mmol L}^{-1}$ , a slight excess compared to ethanol concentration (taking into account process stoichiometry). Excessive  $\beta$ -NAD<sup>+</sup> concentrations could decrease the NADH oxidation current due to the inhibitory effect of high cofactor concentration [13].

### 3.3. Analytical characteristics of ADH biosensor

For constructed biosensor analytical characteristics in ethanol solution were evaluated by chronoamperometric measurements at a potential of  $-50$  mV. Biosensor response displayed linear dependence on the ethanol concentration from  $0.05$  to  $1.1 \text{ mmol L}^{-1}$  with sensitivity of  $2.24 \text{ } \mu\text{A L mmol}^{-1}$  ( $8.0 \text{ } \mu\text{A L}^{-1} \text{ mmol}^{-1} \text{ cm}^2$ ). The limit of detection estimated according to  $3 S_x/a$  rule (see 3.1) was  $25 \text{ } \mu\text{mol L}^{-1}$ . It is apparent (see Table 1) that the linear range compares reasonably well with other reported biosensors. The sensitivity of the proposed biosensor is substantially higher, indicating that ADH immobilized in TiO<sub>2</sub>/MB/MWCNT/Nafion nanocomposite has greater catalytic activity. The limit of detection calculated is comparable with those reported for an ordered mesoporous carbon/MB glassy carbon electrode,  $19 \text{ } \mu\text{mol L}^{-1}$  [9], and for tetrathiafulvalene and cysteamine immobilized in a form of self-assembled monolayer on a gold electrode,  $30 \text{ } \mu\text{mol L}^{-1}$  [1]. It is lower than the  $50 \text{ } \mu\text{mol/L}$  obtained from GCE modified with poly(nile blue A) and SWCNT [12], and significantly better than both the  $100 \text{ } \mu\text{mol L}^{-1}$  reported for SWCNT functionalized with poly(brilliant cresyl blue) [8] and the  $300 \text{ } \mu\text{mol L}^{-1}$  evaluated for the 1,10-phenantroline-5,6-dione/MWCNTs electrode [5]. However, it is not as good as those reported for biosensors with ADH entrapped in

a MWCNT/MB matrix,  $6 \mu\text{mol L}^{-1}$  [10], for toluidine blue O covalently attached to a cellulose acetate ADH modified electrode,  $5 \mu\text{mol L}^{-1}$  [3], or for ADH immobilized into  $\text{Au}_{\text{col}}/\text{MWCNT}/\text{Teflon}$  composite,  $4.7 \mu\text{mol L}^{-1}$  [13].

Under optimum conditions the enzymatic reaction at the  $\text{TiO}_2/\text{MB}/\text{MWCNT}/\text{Nafion}$  nanocomposite fitted well into Michaelis-Menten kinetic model. The apparent Michaelis-Menten constant,  $K_m^{\text{app}}$ , an indication of the enzyme kinetics calculated from electrochemical version of the Lineweaver-Burk equation [8], was  $1.24 \text{ mmol L}^{-1}$ . This value is lower than that reported for ADH entrapped into a SWCNT/poly(nile blue A) matrix,  $6.30 \text{ mmol L}^{-1}$  [12]; for an ethanol biosensor based on ADH immobilized in poly(dimethyldiallylammonium chloride)/SWCNT nanocomposite,  $5.0 \text{ mmol L}^{-1}$  [33]; for ADH immobilized into  $\text{Au}_{\text{col}}/\text{MWCNT}/\text{Teflon}$  layer,  $4.95 \text{ mmol L}^{-1}$  [13] and for biosensors based on ADH immobilized into nanocomposite of SWCNT functionalized with poly(brilliant cresyl blue),  $2.3 \text{ mmol L}^{-1}$  [8]. These results indicate that the proposed biosensor possesses high biological affinity for ethanol.

Biosensor repeatability was evaluated for five electrodes in  $0.3 \text{ mmol L}^{-1}$  ethanol solution; relative standard deviations varied from 1.4% to 4.6%. For a series of five electrodes prepared at different times, RSD of 7.3% was obtained for  $0.3 \text{ mmol L}^{-1}$  ethanol solution. These results are considered satisfactory in terms of repeatability.

Operational stability was examined by thirty successive measurements under optimum conditions. Repetitive chronoamperometric measurements performed in  $0.3 \text{ mmol L}^{-1}$  ethanol solution showed no significant signal change, with recorded current values between 97 and 104% of the mean of the first three measurements (see Fig. 5) which constitutes an acceptable level of reproducibility. A less attractive feature of the constructed biosensor was its long-term stability. After one week of storage in buffer solution at  $4^\circ\text{C}$  the biosensor retained only 54% of its initial current response and after an additional week, 48%. This response decrease may be attributed to enzyme leaching from the matrix and the inherent instability of ADH.

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## 3.4. Analysis of alcoholic beverages

Biosensor performance was checked by determining ethanol content in real samples of alcoholic beverages: wine, liqueur and vodka. From Table 2 it may be observed that the obtained results are in good agreement with alcohol contents reported by producers. Relatively good accuracy of ethanol determination in real alcoholic beverages indicated that measurements carried out at low potential of  $-50 \text{ mV}$  allowed detection of ethanol without interferences from other electroactive compounds usually present in real samples. These results suggest that the ADH/titania sol-gel/MB/MWCNT/Nafion biosensor could be employed for evaluation of ethanol concentrations in alcoholic beverages.

## 4. Conclusion

In this work, an ethanol biosensor was developed by incorporation of ADH, a redox mediator – Meldola's Blue, multi-walled carbon nanotubes and Nafion into a  $\text{TiO}_2$  layer obtained by a sol-gel technique. Optimal biosensor matrix composition was determined by varying the amount of MWCNT, Nafion and enzyme in the biolayer under controlled experimental conditions such as temperature and pH of background electrolyte. The electrocatalytic effect of CNT, the electron-mediator properties of MB, and an appropriate environment of sol-gel matrix mixed with Nafion resulted in an ethanol biosensor with good sensitivity, low limit of detection and good operational stability. Nevertheless, the lifetime of the developed biosensor was not satisfactory, suggesting further research. An important advantage of constructed biosensor is the low applied potential of its working electrode,  $-50 \text{ mV}$  (vs.  $\text{Ag}/\text{AgCl}$ ). For a robust biosensing system, the operation potential range of  $-100$  to  $0 \text{ mV}$  is regarded as the ideal region in which most electroactive biological substances do not interfere [9]. The described results demonstrate that the proposed biosensor can be employed for rapid determination of ethanol in real samples of alcoholic beverages.

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